Reference: *Biol. Bull.*, **150**: 402–410. (June, 1976)

GAMETE-RELEASE BY 1-METHYLADENINE *IN VITRO* IN THE SEA CUCUMBER, *LEPTOSYNAPTA INHAERENS*

SUSUMU IKEGAMI, HARUO KANATANI AND SAMUEL S. KOIDE

Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113; Ocean Research Institute, University of Tokyo, Nakano-ku, Tokyo 164; The Population Council, The Rockefeller University, New York, New York 10021 and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

It has been well established that a gonad-stimulating substance released from the nervous tissue induces shedding of gametes and oocyte maturation in starfish (Chaet and McConnaughy, 1959; Kanatani, 1964; Kanatani, Ikegami, Shirai, Oide and Tamura, 1971). The action of the gonad-stimulating substance has been shown to be indirect and is mediated by a second substance, the maturation-inducing substance, which triggers oocyte maturation and shedding of both oocytes and sperm (Schuetz and Biggers, 1967; Kanatani and Shirai, 1967). The maturation-inducing substance is produced in the follicle cells surrounding the oocytes under the influence of the gonad-stimulating substance (Hirai, Chida and Kanatani, 1973). The maturation-inducing substance has been isolated and identified as 1-methyladenine (Kanatani, Shirai, Nakanishi and Kurokawa, 1969).

In the case of the sea cucumber, *Parastichopus californicus,* Strathmann and Sato (1968) reported that the percentage of germinal vesicle breakdown in oocytes increased by treatment with an extract of starfish radial nerve. Further, Hufty and Schroeder (1974) showed that ovaries of *Parastichopus* incubated with the starfish radial nerve produce a substance which induces oocyte maturation in the starfish. The substance was chromatographically identical to 1-methyladenine (Hufty and Schroeder, 1974). On the other hand, Stevens (1970) reported that 1-methyladenine failed to induce spawning in the same sea cucumber.

Since the role of 1-methyladenine in sea cucumber reproduction remains unclear, the present study was carried out in order to determine the effect of 1-methyladenine on the induction of gamete-shedding in the sea cucumber, *Leptosynapta inhaerens.* The effects of related compounds and acetylcholine were also investigated.

A preliminary report (Koide, Ikegami and Kanatani, 1975) of this work was presented at the general meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts.

Materials and Methods

The present experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, during June and July of 1974 and 1975. The sea cucumbers, *Leptosynapta inhaerens,* supplied by the Laboratory were kept in aquaria containing clean sand provided with running sea water.

The gonads were isolated in sea water and cut into pieces. The gonads gen-

erally contained motile spermatozoa and immature oocytes. However, some gonads were full of mature oocytes, and presumably spermatozoa were already shed.

The following compounds were used in order to determine their gamete-shedding activity: 1-methyladenine, 1-methyladenosine, procaine hydrochloride, adenine hydrochloride, guanine, 6-methylaminopurine, 7-methylguanine (Sigma Chemical Co., St. Louis, Missouri), 1-ethyladenine, 7-methyladenine (gifts from Dr. Nakanishi), 6-dimethylaminopurine (Cyclo Chemicals, Los Angeles), acetylcholine hydrochloride (Matheson, Coleman and Bell, Norwood, Ohio), atropine sulfate (Vitarine Co., New York) and *d*-tubocurarine hydrochloride (Abbott Laboratories, North Chicago). They were dissolved in a small amount of distilled water and diluted with sea water or dissolved directly in sea water. The pH of the test solutions was adjusted to 8.0–8.2. The sea water used was Goldstein's artificial sea water (1953).

For the assay of shedding of gametes, small fragments of the gonad which had been kept in artificial sea water were transferred to small Petri dishes containing 1 ml of test solutions, and the degree of gamete-shedding was observed for five minutes. In some cases, observation was continued for one hour to see whether germinal vesicle breakdown of immature oocytes within the gonad as well as spawning were induced.

The effects of atropine sulfate, *d*-tubocurarine hydrochloride and procaine hydrochloride on the gonadal fragments were also investigated in the absence and presence of 1-methyladenine or acetylcholine. Gonadal fragments pretreated with atropine, *d*-tubocurarine or procaine for 20 minutes were transferred to the same test solution containing 1-methyladenine (5×10^{-6} M) or acetylcholine (5×10^{-6} M).

Unless otherwise specified, at least four assays were carried out with isolated gonadal fragments obtained from different individuals at room temperature (about 23° C).

For histological study, samples were fixed in a fixative with the following composition: 5 ml of 50% glutaraldehyde, 25 ml of 8% paraformaldehyde, 20 ml of 0.2 M sodium phosphate buffer (pH 7.4) and 4 g of sucrose. They were post-fixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Sections were made at one μm and stained with toluidine blue.

Results

Response of gonadal fragments to various purine derivatives

Among the purine derivatives tested, 1-methyladenine and 1-ethyladenine were effective in inducing shedding of sperm in isolated gonadal fragments. When the fragments were transferred to sea water containing 1-methyladenine or 1-ethyladenine at a concentration of 5×10^{-6} M, they immediately contracted, resulting in an intensive shedding of sperm (Fig. 1). Subsequently the fragments relaxed gradually and reverted to the original length. It was noted that untreated control fragments showed slow spontaneous rhythmic contractions and relaxations without any shedding of sperm. Other purine derivatives, adenine, guanine, 6-methylamino-

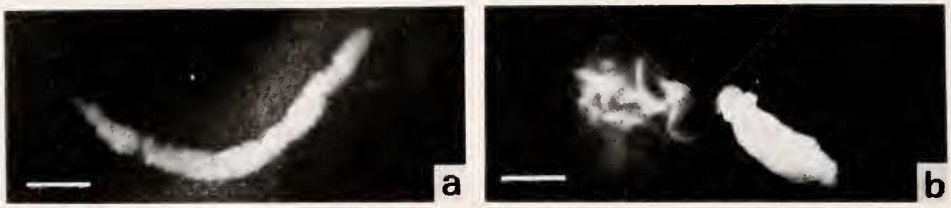

FIGURE 1. Sperm-shedding of an isolated gonadal fragment of *Leptosynapta inhaerens* treated with 1-methyladenine: *a* shows a gonadal fragment before treatment with 1-methyladenine; *b*, the same fragment, contracting and shedding sperm immediately after the treatment with 1 methyladenine at the concentration of 5×10^{-6} M. Scale bars equal 1 mm.

purine, 7-methyladenine, 7-methylguanine, 6-dimethylaminopurine and 1-methyladenosine, failed to induce sperm-shedding at the concentration of 5×10^{-6} M.

It should be pointed out that both 1-methyladenine and 1-ethyladenine were unable to induce spawning (Fig. 2) or maturation (germinal vesicle breakdown) of immature oocytes in the gonad. However, some gonadal fragments which contained mature oocytes whose germinal vesicle had already disintegrated responded immediately to 1-methyladenine (5×10^{-6} M) by shedding of these mature oocytes from the cut surface. Gonads filled with mature oocytes could be obtained from only two individuals during the period of study.

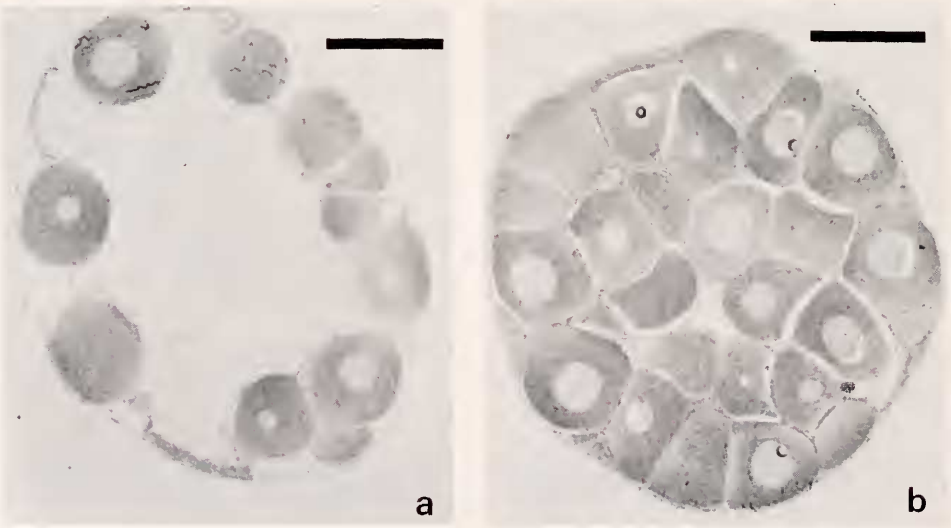

FIGURE 2. Sperm-shedding of an isolated gonadal fragment of *Leptosynapta inhaerens* treated with 1-methyladenine: *a* shows cross section of a gonadal fragment without 1-methyladenine application, showing spermatozoa in the central region and immature oocytes in the peripheral region; *b*, cross section of a gonadal fragment obtained from the same individual as *a*, which was fixed immediately after the application of 1-methyladenine (5×10^{-6} M). Scale bars equal 20 μm.

TABLE I

Effects of 1-methyladenine and 1-ethyladenine on shedding of sperm in vitro *in* Leptosynapta inhaerens *gonad.**

Experiment number†	Chemical	Concentrations					Control
		1.3×10^{-6} M	6.3×10^{-7} M	3.1×10^{-7} M	1.6×10^{-7} M	7.8×10^{-8} M	
1	1-Methyladenine	++	++	−	−	−	−
	1-Ethyladenine	++	±	−	−	−	−
2	1-Methyladenine	++	++	++	++	−	−
	1-Ethyladenine	++	++	++	−	−	−
3	1-Methyladenine	++	++	+	±	−	−
	1-Ethyladenine	++	+	±	−	−	−
4	1-Methyladenine	++	++	++	−	−	−
	1-Ethyladenine	++	++	±	−	−	−

* The degree of sperm-shedding was expressed as follows: ++, intensive shedding; +, intermediate shedding; ±, slight shedding; −, no shedding.

† In the same experiment number, gonads obtained from the same individual were used.

Effects of 1-methyladenine and 1-ethyladenine at various concentrations

Table I shows the effect of 1-methyladenine at various concentrations on shedding of sperm in *Leptosynapta* gonadal fragments. 1-Methyladenine was effective at concentrations above 6.3×10^{-7} M, while with various starfish species it was effective in inducing spawning of ovarian fragments and maturation of oocytes at concentrations greater than 3×10^{-7} M (Kanatani, 1969; Kanatani and Shirai, 1971). These results suggest that the minimal effective dose of 1-methyladenine required for inducing shedding of sperm in *Leptosynapta* gonad is equivalent to the dose necessary for spawning and oocyte maturation in the starfish.

1-Ethyladenine was also effective in inducing shedding of sperm in *Leptosynapta* gonads, although it was less potent than 1-methyladenine (Table I). There was a marked variation in the sensitivity of *Leptosynapta* gonads to the inducing substances when specimens of gonads taken from different individuals were used. The results were more consistent when gonads from the same individuals were used. In the present study, potencies of 1-methyladenine and 1-ethyladenine were compared on the gonadal fragments obtained from the same individual. The minimum effective concentration of 1-ethyladenine was about twice as high as that of 1-methyladenine. Again, these results are similar to those obtained with starfish ovaries with respect to the effectiveness of 1-ethyladenine and 1-methyladenine (Kanatani and Shirai, 1971).

Effects of acetylcholine

Since the time required for inducing shedding of sperm after the application of 1-methyladenine in *Leptosynapta* gonads is very short and shedding occurs within

TABLE II

Comparison of effects of 1-methyladenine and acetylcholine on shedding of sperm in vitro in Leptosynapta inhaerens *gonad.**

Experiment number†	Chemical‡	Concentration, M									Control
		5×10^{-6}	2.5×10^{-6}	1.3×10^{-6}	6.3×10^{-7}	3.1×10^{-7}	1.6×10^{-7}	7.8×10^{-8}	3.9×10^{-8}	2.0×10^{-8}	
1	1-Ma	++	++	++	++	++	−	−	−	−	−
	Ach	++	++	++	±	−	−	−	−	−	−
2	1-Ma	++	++	++	++	++	++	++	+	−	−
	Ach	++	++	++	++	++	++	++	±	−	−
3	1-Ma	++	++	++	+	−	−	−	−	−	−
	Ach	++	−	−	−	−	−	−	−	−	−
4	1-Ma	++	++	++	+	−	−	−	−	−	−
	Ach	++	+	±	−	−	−	−	−	−	−

* Degree of shedding; see Table I.

† In the same experiment number, gonads obtained from the same individual were used.

‡ Abbreviations: 1-Ma, 1-Methyladenine; Ach, Acetylcholine.

five seconds, the action of 1-methyladenine resembles that of acetylcholine in shedding of gametes in sea urchins. Of the neurotransmitters studied, only acetylcholine has been convincingly demonstrated to participate in neurotransmission in sea cucumbers (Welsh, 1966). The present study was, therefore, extended to observe the effect of acetylcholine on the shedding of sperm in *Leptosynapta*. As shown in Table II, acetylcholine at concentrations above 5×10^{-6} M was found to induce instant shedding of sperm as was shown with 1-methyladenine. The minimum effective dose of acetylcholine ranged from 5×10^{-6} to 3.9×10^{-8} M depending on the material used. When compared with the action of 1-methyladenine on gonadal fragments obtained from the same individual, acetylcholine was always less effective than 1-methyladenine and 2- to 4-fold higher concentrations were required to effect shedding of sperm.

Inhibitory effect of d*-tubocurarine on 1-methyladenine and acetylcholine-induced sperm-shedding*

In order to determine the mode of action of 1-methyladenine and acetylcholine on *Leptosynapta* gonad, several drugs were examined for their ability to influence the action of 1-methyladenine or acetylcholine on gonads. It was found that atropine sulfate (10^{-5} M), procaine hydrochloride (10^{-4} M) and *d*-tubocurarine hydrochloride (10^{-4} to 6.3×10^{-6} M) did not induce shedding of sperm. After 20 minutes, the treated fragments were transferred to solutions of 1-methyladenine (5×10^{-6} M) or acetylcholine (5×10^{-6} M) containing the above drugs at the same concentrations as those used in the pretreatment solutions. The fragments pretreated with atropine or procaine shed sperm instantly as the untreated control

did. *d*-Tubocurarine at greater than 5×10^{-5} M inhibited the action of 1-methyladenine or acetylcholine (Table III). The effective concentration of *d*-tubocurarine required to block shedding of sperm induced by 1-methyladenine was the same as that required to inhibit acetylcholine-induced shedding of sperm. The addition of a few drops of 0.5 M KCl to the medium containing the gonadal fragments and *d*-tubocurarine resulted in an instant shedding of sperm. These results suggest that the contractile elements of the fragments remained intact after treatment with *d*-tubocurarine.

Discussion

The results of the present study show that 1-methyladenine, 1-ethyladenine and acetylcholine induced instant shedding of sperm from *Leptosynapta* gonad. However, the effects of these compounds on the sea cucumber gonads differ in some respects from those observed with starfish gonads. The shedding of oocytes or spermatozoa from fragments of starfish gonads in response to 1-methyladenine occurs about 20 to 60 minutes after the treatment. Moreover, germinal vesicle breakdown in the spawned oocytes takes place simultaneously with the spawning (Kanatani and Shirai, 1970). It is noteworthy, however, that the purine bases

Table III

Effect of d-*tubocurarine on 1-methyladenine- and acetylcholine-induced shedding of sperm* in vitro *in* Leptosynapta inhaerens *gonad.* The gonadal fragments were pretreated with* d-*tubocurarine at various concentrations for twenty minutes, and transferred to a solution containing* d-*tubocurarine at the same concentration as that present in the pretreatment medium and 1-methyladenine* (5×10^{-6} M) *or acetylcholine* (5×10^{-6} M).

Experiment number†	Inducing agent	Concentration of *d*-tubocurarine					Control
		10^{-4} M	5×10^{-5} M	2.5×10^{-5} M	1.3×10^{-5} M	6.3×10^{-6} M	
1	1-Methyladenine	−	−	−	−	++	++
	Acetylcholine	−	−	−	++	++	++
2	1-Methyladenine	−	−	−	±	+	++
	Acetylcholine	−	−	−	−	++	++
3	1-Methyladenine	−	−	−	++	++	++
	Acetylcholine	−	−	−	++	++	++
4	1-Methyladenine	−	−	++	++	++	++
	Acetylcholine	−	±	++	++	++	++
5	1-Methyladenine	−	−	−	++	++	++
	Acetylcholine	−	−	++	++	++	++
6	1-Methyladenine	−	−	−	++	++	++
	Acetylcholine	−	−	++	++	++	++

* Degree of shedding; see Table I.

† In the same experiment number, gonads of the same individual were used.

which induce sperm-shedding of *Leptosynapta* gonad also effect spawning and oocyte maturation in various starfish, whereas the purines inactive in inducing sperm-shedding of *Leptosynapta* were without effects on starfish gonads (Kanatani and Shirai, 1971).

Kanatani (1974) has detected the presence of 1-methyladenine in the gonads of the sea urchins, *Pseudocentrotus depressus, Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* and the sand dollars, *Clypeaster japonicus* and *Peronella japonica*. Thus, evidence is gradually accumulating indicating the ubiquitous occurrence of 1-methyladenine in the gonads of Echinodermata.

Kanatani (1974) reported that 1-methyladenine treatment increased the percentage of germinal vesicle breakdown in *Anthocidaris* oocytes. In the sea urchin, *Strongylocentrotus purpuratus*, 1-methyladenine induced shedding of sperm and mature oocytes within a brief period of treatment (R. C. Cochran, University of California at Los Angeles, personal communication). As shown in this study, 1-methyladenine effectively induced shedding of sperm of *Leptosynapta* gonads but was unable to induce spawning and germinal vesicle breakdown of immature oocytes. These results suggest the possibility that a factor(s) other than 1-methyladenine may participate in the induction of oocyte maturation in sea cucumbers. However, it is also possible that the immature oocytes with which the present investigation was carried out were at an early stage of development and were incapable of responding to 1-methyladenine. It was observed with starfish that oocytes must attain a certain cell size before 1-methyladenine can be effective.

In a survey of gonads from more than one hundred *Leptosynapta inhaerens* during the breeding season, only two animals had gonads filled with mature eggs and few, if any, sperm. When these gonads were treated with 1-methyladenine, they shed their eggs instantly as was observed with sperm of other gonadal fragments. This finding indicates that 1-methyladenine may induce shedding of both mature eggs and sperm from gonads.

A failure to cause spawning of immature oocytes by the application of 1-methyladenine may be due to the presence of a thick follicular envelope which adheres oocytes together. In starfish, the disintegration of follicle cells occurs concomitantly with oocyte maturation; thus the matured oocytes are freed from adherence to each other and to the inner surface of the gonadal wall (Kanatani and Shirai, 1970). The detachment of oocytes from each other and from the gonadal wall seems to be a prerequisite for 1-methyladenine-induced spawning in the *Leptosynapta* gonad.

Acetylcholine is known to induce shedding of eggs and sperm in the sea urchin gonads (Iwata and Fukase, 1964). The occurrence and functional role of acetylcholine in sea cucumbers have been well established. Welsh (1954) demonstrated that the longitudinal body wall muscles in sea cucumbers were unusually sensitive to acetylcholine. In addition, acetylcholine-like activity was detected in the longitudinal muscles, intestine and radial nerves of the sea cucumbers, *Parastichopus californicus* and *Holothuria tubulosa* (Welsh, 1966).

Although further studies are required to determine the exact site of action of 1-methyladenine and acetylcholine before any definite conclusion can be drawn on the role of these effectors in shedding of gametes from the *Leptosynapta* gonads, the inhibition of sperm-shedding induced by 1-methyladenine or acetylcholine by

d-tubocurarine suggests that 1-methyladenine acts on nerve cells or at the neuromuscular junction rather than directly on the muscles. Future investigations on the contraction of gonads and shedding of gametes induced with 1-methyladenine will provide new insight into the mechanism of action of 1-methyladenine in the reproductive physiology of Echinodermata.

We thank Dr. K. Selman of University of Florida and Dr. J. Kubota of University of Tokyo for preparing histological sections; Dr. J. M. Arnold of University of Hawaii for taking photographs; and Dr. C. L. Prosser of University of Illinois, Dr. W. M. Yau of Southern Illinois University and Dr. D. M. Phillips of the Population Council for helpful discussions. Thanks are also due to Dr. K. Nakanishi of Columbia University and Dr. Y. Iwanami of Sasaki Institute in supplying some of the chemicals used in this study.

This investigation was supported in part by a Biomedical fellowship to S. I. and a travel grant (MC75.06C) to H. K. from the Population Council, New York.

Summary

1. The hermaphroditic sea cucumber *Leptosynapta inhaerens* does contain mature spermatozoa and immature oocytes during the breeding season. In some animals, the gonad contains eggs and few, if any, sperm.

2. Isolated fragments of ovotestes of the sea cucumber were found to shed sperm immediately upon treatment with 1-methyladenine at concentrations higher than 6.3×10^{-7} M.

3. 1-Methyladenine failed to induce both spawning and maturation of immature oocytes within the gonadal fragments, and was unable to effect germinal vesicle breakdown. Treatment of gonadal fragments containing *mature* oocytes with 1-methyladenine caused a release of these oocytes as was observed with sperm.

4. 1-Ethyladenine was also effective in inducing shedding of sperm at concentrations higher than 1.3×10^{-6} M. The following purine derivatives had no sperm-shedding effect at the concentration of 5×10^{-6} M: adenine, guanine, 6-methylaminopurine, 7-methyladenine, 7-methylguanine, 6-dimethylaminopurine and 1-methyladenosine.

5. Acetylcholine hydrochloride at concentrations greater than 5×10^{-6} M induced shedding of sperm. The sperm-shedding action of both 1-methyladenine (5×10^{-6} M) and acetylcholine (5×10^{-6} M) was completely inhibited in the presence of *d*-tubocurarine hydrochloride at concentrations higher than 5×10^{-5} M.

6. The present results suggest that 1-methyladenine acts on the gonadal nerve cells or at the neuromuscular junction of *Leptosynapta* gonads to induce contraction of the gonad, resulting in gamete-shedding.

LITERATURE CITED

Chaet, A. B., and R. A. McConnaughy, 1959. Physiologic activity of nerve extracts. *Biol. Bull.*, **117**: 407–408.

Goldstein, L., 1953. A study of the mechanism of activation and nuclear breakdown in the *Chaetopterus* egg. *Biol. Bull.*, **105**: 87–102.

Hirai, S., K. Chida, and H. Kanatani, 1973. Role of follicle cells in maturation of starfish oocytes. *Develop. Growth Differ.*, **15**: 21–31.

Hufty, H. M., and P. C. Schroeder, 1974. A hormonally active substance produced by the ovary of the holothurian *Parastichopus californicus*. *Gen. Comp. Endocrinol.*, **23**: 348–351.

Iwata, K. S., and H. Fukase, 1964. Artificial spawning in sea urchins by acetylcholine. *Biol. J. Okayama Univ.*, **10**: 51–56.

Kanatani, H., 1964. Spawning of starfish: Action of gamete-shedding substance obtained from radial nerves. *Science*, **146**: 1177–1179.

Kanatani, H., 1969. Induction of spawning and oocyte maturation by 1-methyladenine in starfishes. *Exp. Cell Res.*, **57**: 333–337.

Kanatani, H., 1974. Presence of 1-methyladenine in sea urchin gonad and its relation to oocyte maturation. *Develop. Growth Differ.*, **16**: 159–170.

Kanatani, H., and H. Shirai, 1967. *In vitro* production of meiosis inducing substance by nerve extract in ovary of starfish. *Nature*, **216**: 284–286.

Kanatani, H., and H. Shirai, 1970. Mechanism of starfish spawning. III. Properties and action of meiosis-inducing substance produced in gonad under influence of gonad-stimulating substance. *Develop. Growth Differ.*, **12**: 119–140.

Kanatani, H., and H. Shirai, 1971. Chemical structural requirements for induction of oocyte maturation and spawning in starfishes. *Develop. Growth Differ.*, **13**: 53–64.

Kanatani, H., H. Shirai, K. Nakanishi, and T. Kurokawa, 1969. Isolation and identification of meiosis inducing substance in starfish *Asterias amurensis*. *Nature*, **221**: 273–274.

Kanatani, H., S. Ikegami, H. Shirai, H. Oide, and S. Tamura, 1971. Purification of gonad-stimulating substance obtained from radial nerves of the starfish, *Asterias amurensis*. *Develop. Growth Differ.*, **13**: 151–164.

Koide, S. S., S. Ikegami, and H. Kanatani, 1975. Gamete release from isolated sea cucumber gonads by 1-methyladenine. *Biol. Bull.*, **149**: 433–434.

Schuetz, A. W., and J. D. Biggers, 1967. Regulation of germinal vesicle breakdown in starfish oocytes. *Exp. Cell Res.*, **46**: 624–628.

Stevens, M., 1970. Procedures for induction of spawning and meiotic maturation of starfish oocytes by treatment with 1-methyladenine. *Exp. Cell Res.*, **59**: 482–484.

Strathmann, R. R., and H. Sato, 1968. Increased germinal vesicle breakdown in oocytes of the sea cucumber *Parastichopus californicus* induced by starfish radial nerve extract. *Exp. Cell Res.*, **54**: 127–129.

Welsh, J. H., 1954. Marine invertebrate preparations useful in the bioassay of acetylcholine and 5-hydroxytryptamine. *Nature*, **173**: 955–956.

Welsh, J. H., 1966. Neurohumors and neurosecretion. Pages 545–560 in R. A. Boolootian, Ed., *Physiology of Echinodermata*. Interscience Publishers, New York.